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Pathways of caspase-mediated apoptosis in autosomal-dominant polycystic kidney disease (ADPKD)

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Pathways of caspase-mediated apoptosis in autosomal-dominant polycystic kidney disease (ADPKD).

Background. We have recently demonstrated an increase in apoptosis in Han:SPRD rat kidneys with autosomal-dominant polycystic kidney disease (ADPKD). Caspase-3 and caspase-7 are major mediators of apoptosis. There are two pathways of caspase-3 and caspase-7–mediated apoptosis: (*1*) the "extrinsic" pathway involving the death receptor Fas, Fas ligand (FasL), and caspase-8 and (*2*) the "mitochondrial" or "intrinsic" pathway involving Bcl-2 proteins, caspase-2, cytochrome c release, and caspase-9. The aim of the present study was to investigate the pathways of apoptosis in 3-week-old Han:SPRD rats with ADPKD.

Methods. Fluorescent substrates were used to measure caspase activity. mRNA and protein was determined by ribonuclease protection assays and immunoblotting, respectively. The effect of caspase inhibitors on caspase activity in polycystic kidneys was determined.

Results. Caspase-3 and caspase-7 activity was more than 100% increased in homozygous (Cy/Cy) compared to heterozygous (Cy/+) and normal littermate control (+/+) kidneys. Ribonuclease protection assays demonstrated no difference in caspase-3 mRNA. On immunoblotting, there was an increase in the proform of caspase-3 and caspase-7 in Cy/Cy compared to +/+ and Cy/+ kidneys. Caspase-8 and caspase-9 activity was more than 100% increased in Cy/Cy compared to Cy/+ and +/+ kidneys. On immunoblotting, there was an increase of the proform of both caspase-8 and caspase-9 in Cy/Cy kidneys. There was also an increase in cytochrome c release into the cytosol and an increase in caspase-2 protein and activity in Cy/Cy kidneys. On ribonuclease protection assay there was no difference in FasL mRNA between +/+, Cy/+, and Cy/Cy kidneys. Short-term treatment of Cy/Cy rats with the caspase inhibitor IDN-8050 resulted in inhibition of caspase-3 and caspase-7 activity in the kidney.

Conclusion. In Cy/Cy kidneys with ADPKD, there was an increase of the proform of caspase-9, an increase in cytochrome c release into the cytosol, and an increase in caspase-2 protein and activity demonstrating involvement of the intrinsic pathway. There was an increase in the proform of caspase-8 demonstrating involvement of the extrinsic pathway. No differences in FasL mRNA were seen suggesting that the extrinsic pathway is independent of the death receptor ligand, FasL.

Key words: caspase-8, caspase-9, caspase-7, cytochome c, caspase-2, Han:SPRD rat.

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Apoptosis is a pathologic feature of polycystic kidney disease (PKD) [1]. Increased levels of apoptosis are observed in human autosomal-dominant PKD (ADPKD) [1–4] and dysplastic renal disease [5]. Increased apoptosis is also a feature of animal models of PKD, including the cpk mouse model of autosomal-recessive PKD (ARPKD) [3], transgenic mice overexpressing the proto-oncogene c-myc (SBM mice) [6], mice lacking the transcription factor activating protein 2β (AP-2β) [7], Bcl-2–deficient mice [8], and the Han:SPRD rat [9]. Apoptosis was detected in kidneys of humans with ADPKD regardless of renal function, but not in normal kidneys [3]. Mutations of PKD1 are responsible for approximately 85% of cases of ADPKD. A recent study suggests that polycystin-1, the gene product of PKD1, may regulate apoptosis during spontaneous tubulogenesis in Madin-Darby canine kidney (MDCK) cells [10].

Both caspase-3 and caspase-7 play a crucial and extensively studied role in the promotion of all forms of apoptotic cell death [11]. There are two major pathways of caspase-mediated apoptosis [12] (Fig. 1). In the mitochondrial or "intrinsic" pathway, stress-induced signals act via Bcl-2 proteins to cause cytochrome c release from mitochondria. Caspase-2 is a recently discovered caspase that is also a critical initiator of the mitochondrial apoptosis pathway [13]. Cytochrome c binds to the cytosolic protein, apoptosis protease-activating factor-1 (APAF-1), which recruits and activates caspase-9. Active caspase-9 in turn recruits and activates the "executioners" procaspase-3 and procaspase-7. In the "extrinsic" pathway, the binding of a ligand to its death receptor recruits an adaptor protein that in turn recruits and activates procaspase-8. For example, Fas ligand (FasL) binds to its death receptor Fas that recruits an adaptor

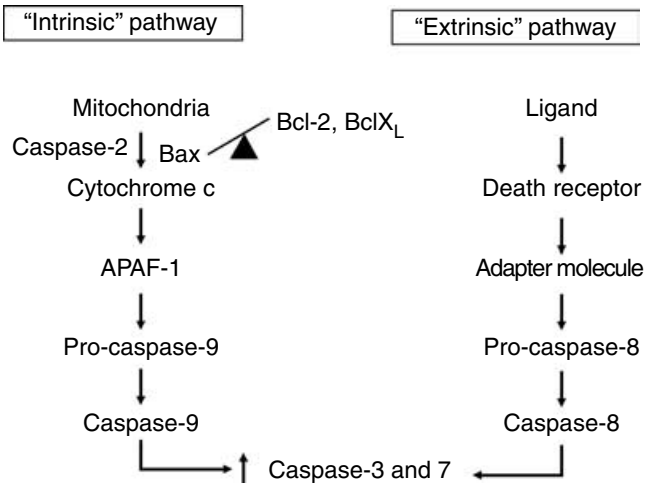

Fig. 1. Major pathways of caspase-3–mediated apoptosis. In the mitochondrial pathway, the balance of pro- and antiapoptotic Bcl-2 family proteins as well as caspase-2 determines cytochrome c release from mitochondria. Cytochrome c binds to the cytosolic protein, apoptosis protease-activating factor-1 (APAF-1), which recruits caspase-9. Caspase-9 in turn activates the "executioner" procaspase-3. In the death receptor pathway, the binding of a ligand (e.g. Fas ligand) to its death receptor recruits an adaptor protein that in turn recruits procaspase-8.

protein called Fas-associated death domain (FADD). FADD in turn recruits and activates procaspase-8. We have recently demonstrated a decrease in the antiapoptotic Bcl-X_L protein and an increase in caspase activity and apoptosis in 2-week-old Han:SPRD rat kidneys with ADPKD [9].

The molecular pathways of caspase-mediated apoptosis, however, have not been characterized in the Han:SPRD rat. The Han:SPRD rat closely resembles ADPKD and therefore is a worthy model to study the molecular pathways of apoptosis. In the present study, therefore, the extrinsic and intrinsic pathways of caspase-mediated apoptosis were investigated.

METHODS

Animals

The study was conducted in 3-week-old normal (+/+), heterozygous (Cy/+), and homozygous (Cy/Cy) Han:SPRD rats. The Cy/Cy Han:SPRD rats died at 4 weeks of age from massive polycystic kidneys and renal failure [14]. All the normal rats and Cy/+ rats studied were males. The Cy/Cy rats studied were both male and female. A colony of Han:SPRD rats was established in our animal care facility from a litter that was obtained from the Polycystic Kidney Program at the University of Kansas Medical Center, Kansas City, Kansas. The study protocol was approved by the University of Colorado Health Sciences Center Animal Care and Use Committee. Rats had free access to tap water and standard rat chow.

Tissue preparation

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and kidneys were removed. The kidneys were weighed after removal. The left kidney was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 120 minutes and then put into 70% ethanol for histologic examinations. The right kidney was frozen in liquid nitrogen and stored at –80°C for ribonuclease protection assays, caspase assays, and Western blot analyses.

Blood was drawn by cardiac puncture. Serum creatinine was measured using a Beckman autoanalyzer (Beckman Instruments, Fullerton, CA, USA).

Morphologic analysis of cystic disease

Tissues were fixed in 4% paraformaldehyde. Four micrometer transverse sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS). Measurements were be made in renal cortex in the Cy/+ rats because the cystic disease mainly involves the cortex [14]. Cyst volume density was scored in histologic sections projected onto a standard grid using point counting stereology as previously described [15]. Briefly, the presence of cysts at the crossing points on a 10 × 10 division grid were determined. One hundred points in 5 to 10 high power fields of each section were be counted in areas of the cortex at 90, 180, and 270 degrees from the hilum to guard against reviewer bias and field selection variation.

Tissue preparation of cytosolic fraction for cytochrome c immunoblot analysis

The cytochrome c release assay was performed as previously described [16]. Kidneys were rapidly removed from the rats. Half a kidney was immediately homogenized in 1.5 mL of mitochondrial buffer A using 10 strokes of a glass Dounce homogenizer. Buffer A contained 250 mmol/L sucrose, 20 mmol/L HEPES-potassium hydroxide (KOH) (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L sodium ethyleneglycoltetraacetic acid (Na EGTA), 1.5 mmol/L sodium ethylenediaminetetraacetic acid (Na EDTA), 1 mm $MgCl_2$, 1 mmol/L dithiothreitol (DTT) plus proteinase inhibitors: 1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 15 μmol/L pepstatin A, 14 μmol/L L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane (E-64), 40 μmol/L bestatin, 22 μmol/L leupeptin, and 0.8 μmol/L aprotonin. The homogenate was centrifuged at 1000*g* for 10 minutes at 4°C to remove large debris, nuclei, and unbroken cells. Supernatants containing mitochondria were further centrifuged at 40,000*g* for 20 minutes at 4°C. The resulting supernatant was saved as the cytosolic extract.

Caspase assays

The activity of caspases in was determined by use of flourescent substrates as we have previously

described [17] with modifications [18]. Cytosolic extracts were prepared as follows. Renal cortex was mixed with a lysis buffer containing 25 mmol/L Na^+ Hepes, 2 mmol/L DTT, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-pro-panesulfonate (CHAPS), 10% sucrose, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 μmol/L pepstatin A, pH 7.2, and homogenized with 10 strokes in a glass-Teflon homogenizer. The lysate was then centrifuged at 4°C at 100,000*g* in a Beckman Ti70 rotor for 1 hour (Beckman Instruments). The resultant supernatants (cytosolic extracts) were immediately frozen in liquid nitrogen (N_2) and stored at −70°C until use. Lysate protein was measured by the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as standards.

The caspase assay was then performed on this supernatant as follows: 200 μg protein (20 to 50 μL volume) was added to 10 μL of the substrate (final concentration 50 μmol/L). The volume was made up to 200 μL with the caspase assay buffer. The assay buffer for caspase-3 and caspase-7 contained 250 mmol/L K^+ Hepes, 50 mmol/L KCl, 1 mmol/L DTT, 1 mmol/L EDTA, and 0.1% CHAPS, pH 7.4. Ac-Asp-Glu-Val-Asp-7 -amido-4-methyl coumarin (Ac-DEVD-AMC) in 10% dimethyl sulfoxide (DMSO) was used as a susceptible substrate for caspase-3 and caspase-7 [19]. The assay buffer for caspase-8 and caspase-9 contained 75 mmol/L Na^+ 3-[N-morpholino]propane sulfonic acid (MOPS), 10% glycerol, 1 mmol/L DTT, 1 mmol/L EDTA, pH 7.4. Ac-Ile-Glu-Pro-Asp-7-Amino-4-methylcoumarin (IEPD-AMC) in 10% DMSO was used as a susceptible substrate for caspase-8 and caspase-9 [19]. The assay buffer for caspase-2 contained 50 mmol/L Hepes, 100 mmol/L NaCl, 10 mmol/L DTT, 1 mmol/L EDTA, 0.1% CHAPS, and 10% glycerol, pH 7.4. Z-Val-Asp-Val-Ala-Asp-AMC (Z-VDVAD-AMC) was used as a susceptible substrate for caspase-2. The assay buffer for caspase-1 contained 100 mmol/L Hepes, 10% sucrose, 10 mmol/L DTT, 0.1 mmol/L EDTA, and 0.1% CHAPS, pH 7.5. Ac-Tyr-Val-Ala-Asp-AMC (Ac-YVAD-AMC) was used as a susceptible substrate for caspase-1. The solution was preincubated for 10 minutes at 30°C before substrate was added. The reaction was then initiated by addition of substrate. Peptide cleavage was measured over 1 hour at 30°C using a Cytofluor 4000 series fluorescent plate reader (Perseptive Biosystems, Framingham, MA, USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. An AMC standard curve was determined for each experiment. Caspase activity was expressed in nmol AMC released per minute of incubation time per mg of lysate protein.

Caspase activity was also measured in nuclear extracts. Nuclei were isolated by the method of Chauveau, Moule, and Rouiller [20] with modifications [21]. The entire procedure was performed at 4°C. Briefly a whole kidney was minced with a blade and then homogenized in a Dounce homogenizer with a loose fitting pestle in a buffer containing 0.33 mol/L sucrose and 0.004 mol/L $CaCl_2$. The homogenate was filtered through a layer of cheesecloth and centrifuged at 600 × *g* for 10 minutes. The resulting pellet was homogenized in a Dounce homogenizer with a tight fitting pestle in a buffer containing 0.25 mol/L sucrose and 0.003 mol/L $CaCl_2$, underlaid with an equal volume of 0.34 mol/L sucrose and 0.003 mol/L $CaCl_2$ and then centrifuged at 1500*g* for 15 minutes. The pellet was resuspended in 9 volumes of 2.4 mol/L sucrose and 0.003 mol/L $CaCl_2$ by homogenization. The homogenate was then centrifuged at 45,000*g* in a Beckman Ti70 rotor for 1 hour (Beckman Instruments). The pellet was resuspended in 0.25 mol/L sucrose and 0.003 mol/L $CaCl_2$ and centrifuged at 600*g* for 10 minutes. The presence of nuclei in the pellet was confirmed by microscopy demonstrating only rounded nuclei with clear sharp nucleoli and no other particles. The nuclei were resuspended in lysis buffer and the caspase assay was performed as described above.

Immunoblot analysis

Caspase-8 and caspase-9 belong to group III or "initiator" caspases that share the same synthetic substrate specificities [19]. Caspase-3 and caspase-7 belong to group II or "executioner" caspases and also share the same synthetic substrate specificities [19]. The substrates used in fluorescence or colorimetric assays preferentially detect active members of a given caspase group rather than a specific caspases [22]. Thus, immunoblot analysis for specific caspases was undertaken.

Immunoblot analysis was performed as we have previously described [17]. Renal cortex was homogenized in lysis buffer (in mmol/L): 5 Na_2HPO_4, 5 NaH_2PO_4, 150 NaCl, 1 EDTA, 0.1% Triton X-100, 50 NaF, and 0.2 Na_3VO_4, and 0.1% β-mercaptoethanol, pH 7.2, plus proteinase inhibitors 1 mmol/L AEBSF, 15 μmol/L pepstatin A, 14 μmol/L E-64, 40 μmol/L bestatin, 22 μmol/L leupeptin, and 0.8 μmol/L aprotonin. The homogenates were centrifuged (14,000 rpm at 4°C for 10 minutes) to remove unbroken cells and debris. Supernatants were mixed with sample buffer containing 50 mmol/L Tris base (pH 6.8), 0.5% glycerol, 0.01% bromphenol blue, and 0.75% sodium dodecyl sulfate (SDS) and heated at 95°C for 5 minutes. Equal amounts of protein (50 to 100 μg/lane) were fractionated by Tris-glycine-SDS-7.5 or 12.5% polyacrylamide gel electrophoresis (PAGE). The electrophoretically separated proteins were then transferred to an Immobilon P membrane (Millipore, Bedford, MA, USA) by wet electroblotting for 90 minutes. The membranes were blocked with 5% nonfat dry milk in TBST [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween-20] buffer at pH 7.5 for 1 hour at room temperature. Immunoblot analyses were performed with the

following primary antibodies: (*1*) a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 1-277 representing the full length precursor form of caspase-3 of human origin (1:200) (catalogue number sc-7148) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [a purified recombinant caspase-3 (Upstate Group Inc, Lake Placid, NY, USA) was used as a positive control]; (*2*) a rabbit polyclonal antibody that detects the full-length protein of caspase-7 (1:500) (Cell Signaling Technology, Inc., Beverly, MA, USA) [Jurkat whole cell lysate (catalogue number sc-2204) (Santa Cruz Biotechnology, Inc) was used a positive control]; (*3*) a rabbit anticaspase-8 polyclonal antibody that detects the full-length recombinant human caspase-8 protein (1:2000) (catalogue number AAP-118) (StressGen Biotechnologies Corp, Victoria, BC, Canada) (the immunogen for the antibody was full-length recombinant human caspase-8 protein and the antibody has species reactivity to human, mouse, and rat); (*4*) a mouse anticaspase-9 monoclonal antibody that detects the human caspase-9 glutathione-S-transferase (GST) fusion protein fragment corresponding to the N-terminal region, residues 1-250 (1:2000) (catalogue number AAM-139) (StressGen Biotechnologies Corp.); (*5*) purified mouse anticytochrome c monoclonal antibody (1:1000) (catalog number 556433) (BD Pharmingen, San Diego, CA, USA) (RSV-3T3 cell lysate was used as a positive control); and (*6*) a rabbit polyclonal antibody raised against a peptide mapping at the amino terminus of caspase-2 of human origin (1:1000) (catalogue number sc-623) (Santa Cruz Biotechnology, Inc.) (the antibody has species reactivity to human, mouse, and rat) [Jurkat whole cell lysate (catalogue number sc-2204) (Santa Cruz Biotechnology, Inc.) was used a positive control]. The membranes were incubated overnight at 4°C with primary antibodies, washed in TBST buffer and further incubated with sheep antimouse IgG or donkey antirabbit IgG coupled to horseradish peroxidase (Amersham, Piscataway, NJ, USA) at 1:1000 dilution in TBST buffer for 1 hour at room temperature. Subsequent detection was carried out by enhanced chemiluminescence (ECL) (Amersham), according to the manufacturer's instructions. Prestained protein markers (Bio-Rad) were used for molecular mass determination.

Ribonuclease protection assay

FasL, caspase-2, caspase-3, Bax, and Bcl-X_L mRNA levels were analyzed by RNAse protection assays using rAPO-1 multiprobe template (BD Pharmingen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control. Total RNA was prepared form whole kidney using the TRIzol method. mRNA was enriched using a MicroPoly(A)Pure Kit (Ambion, Inc., Austin, TX, USA). Nonradioactive RNA probes were transcribed from a multiprobe template set for rat apoptosis factors (rAPO-1) (Pharmingen). An in vitro RNA transcription kit (MAXIscript T7) (Ambion, Inc.) was applied with the addition of biotin-16-uridine triphosphate (UTP) for labeling the probes. Five micrograms of mRNA enriched samples were incubated with about 600 pg of the probe overnight at 56°C, and then digested with RNase. Protected mRNAs were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel and transferred to a positively charged nylon membrane. Chemiluminescence was generated with streptavidin-conjugated alkaline phosphatase. The membrane was then exposed to a film.

Immunohistochemistry

Immunohistochemical localization of active caspase-3 was performed on paraformaldehyde-fixed and paraffin-embedded tissues. Paraffin blocks were sectioned at 4 μm thickness on slides. Paraffin sections were deparaffinized in xylene and rehydrated in graded ethanols. The sections were then heated to 110°C in 1 mmol/L EDTA in PBS in a cooker (Decloaking Chamber) (Biocare Medical, Walnut Creek, CA, USA) for 10 minutes for antigen retrieval. Sections were incubated with blocking buffer (5% normal goat serum and 2% BSA in PBS) for 30 minutes to block nonspecific binding. Sections were then incubated with antiactive caspase-3 antibody (Promega, Madison, WI, USA) (catalogue number G7481) 1:50 in blocking buffer for 1 hour. Negative control sections were incubated with blocking buffer. Following wash with TBST (10 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween-20), the sections were incubated with horseradish peroxidase (HRP)-labeled polymer (Dako EnVision System, Carpinteria, CA, USA) (catalogue number K1395) for 1 hour. Sections were washed with TBST again and the antigen site was visualized by incubating the sections with the substrate 3,3′-diaminobenzidine (DAB) chromogen. Negative control sections showed no staining with DAB.

Caspase inhibition

The pan caspase inhibitor IDN-8050 was obtained from Idun Pharmaceuticals, Inc. (San Diego, CA, USA). The inhibitor (1 mg) or vehicle [polyethylene glycol 300 (PEG 300)] was administered intraperitoneally. Three hours later, both kidneys were removed, washed with normal saline, frozen in liquid nitrogen, and stored at −80°C for caspase assays.

The caspase-2 inhibitor, Z-Val-Asp(OMe)-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VDVAD-FMK) was obtained from Enzyme Systems Products (Livermore, CA, USA). Cy/Cy rats were injected intraperitoneally with the inhibitor at a dose of 20 mg/kg as recommended by the manufacturer. Three hours later, both kidneys were removed, washed with normal saline, frozen in liquid nitrogen, and stored at –80°C for caspase assays.

Statistical analysis

Nonnormally distributed data were analyzed by the nonparametric unpaired Mann-Whitney test. Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) with post test according to Newman-Keuls. A P value of <0.05 was considered statistically significant. Values are expressed as means ± SE.

RESULTS

Cyst formation and renal function

In 3-week-old rats, cyst volume density (%) was 0.5 ± 0.1 in +/+ and 7.4 ± 1.4 in Cy/+ ($P < 0.05$ vs. +/+) and 76.8 ± 3.2 in Cy/Cy ($P < 0.001$ vs. Cy/+) ($N = 6$). Serum creatinine (mg/dL) was 0.4 ± 0.02 in +/+ and 0.4 ± 0.03 in Cy/+ (P = NS vs. +/+) and 1.1 ± 0.07 in Cy/Cy ($P < 0.001$ vs. Cy/+) ($N = 6$).

In 8-week-old rats, cyst volume density (%) was 0.6 ± 0.2 in +/+ and 34 ± 6 in Cy/+ ($P < 0.0001$ vs. +/+) ($N = 7$). Serum creatinine (mg/dL) was 0.4 ± 0.02 in +/+ and 0.3 ± 0.02 in Cy/+ (P = NS vs. +/+) ($N = 7$).

Caspase-3 and caspase-7 activity

Caspase-3 and caspase-7 activity was measured using the fluorescent substrate DEVD-AMC. Caspase-3 and caspase-7 activity (nmol/min/mg) was 8.8 ± 1.2 in +/+ and 8.5 ± 0.8 in Cy/+ (P = NS vs. +/+) ($N = 11$) and 16.7 ± 1.8 in Cy/Cy rats ($P < 0.01$ vs. +/+ and Cy/+) ($N = 7$) (Fig. 2). We previously demonstrated a slight increase in caspase-3 activity in 2-week-old Cy/+ kidneys compared to +/+ [9]. This increase was not reproducible in the present study in 3-week-old kidneys. As caspase-3 and caspase-7 activity was not increased in Cy/+ rats at 3 weeks, it was determined whether it increased at a later age. Caspase-3 activity was increased in 8-week-old Cy/+ rat kidneys. Caspase-3 activity (nmol/min/mg) in 8-week-old rats was 10 ± 1 in +/+ and 14 ± 1 in Cy/+ ($P = 0.02$) ($N = 7$).

Caspase-3 and caspase-7 activity was also measured in isolated nuclei. Caspase-3 and caspase-7 activity (nmol/min/mg), was 3.5 ± 0.4 in nuclei isolated from +/+ rat kidneys and 4.8 ± 0.4 in Cy/+ (P = NS vs. +/+) ($N = 11$) and 6.6 ± 0.9 in Cy/Cy rats ($P < 0.05$ vs. +/+) (NS vs. Cy+, $N = 4$).

Caspase-3 mRNA

Ribonuclease protection assay for caspase-3, demonstrated no difference in caspase-3 mRNA between Cy/Cy, Cy/+ and normal littermate controls (+/+) (Fig. 3).

Caspase-3 and caspase-7 protein

On immunoblot for caspase-3, there was an increase in the proform (32 kD) in Cy/Cy compared to +/+ and Cy/+ rat kidneys (Fig. 4). On immunoblot for caspase-7, there was an increase in the proform (35 kD) in Cy/Cy compared to +/+ and Cy/+ rat kidneys (Fig. 5). Caspase-7 was not detected in +/+ and Cy/+ kidneys (Fig. 5).

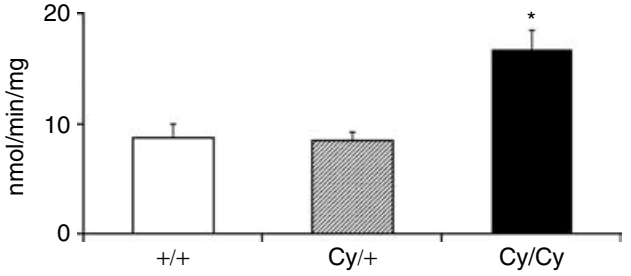

Fig. 2. Caspase-3 and caspase-7 activity in 3-week-old Han:SPRD rat kidneys. Cytosolic extracts of renal cortex were prepared. The activity of caspase-3 and caspase-7 was determined using the fluorescent substrate Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC). There was an increase in caspase-3 and caspase-7 activity in 3-week-old homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys. *$P < 0.01$ vs. +/+ and Cy/+ ($N = 11$).

Caspase-8 and caspase-9 activity

Caspase-8 and caspase-9 activity was measured using the fluorescent substrate IEPD-AMC. Caspase-8 and caspase-9 activity (nmol/min/mg) was 60 ± 10 in +/+ and 72 ± 10 in Cy/+ (P = NS vs. +/+) and 123 ± 7 in Cy/Cy rats ($P < 0.001$ vs. +/+ and Cy/+) ($N = 10$) (Fig. 6). As caspase-8 and caspase-9 activity was not increased in Cy/+ rats at 3 weeks, it was determined whether it increased at a later age. Caspase-8 and caspase-9 activity was increased in 8-week-old Cy/+ rats. Caspase-8 and caspase-9 activity (nmol/min/mg) in 8-week-old rats was 18 ± 2 in +/+ and 25 ± 0.5 in Cy/+ ($P < 0.05$ vs. +/+) ($N = 4$).

Caspase-8 and caspase-9 protein

As IEPD-AMC does not differentiate between caspase-8 and caspase-9, immunoblot analysis was undertaken. There was a massive increase in the pro-form of caspase-8 (55 kD) in Cy/Cy vs. +/+ and Cy/+ kidneys (Fig. 7). In Cy/Cy kidneys there was an increase in the proform (46 kD) of caspase-9 (Fig. 8).

Mitochondrial release of cytochrome c

Release of cytochrome c from mitochondria is an essential factor in the activation of caspase-9 [23]. An increase in cytochrome c (15 kD) in the cytosol was detected on immunoblot analysis in Cy/Cy compared to +/+ and Cy/+ kidneys (Fig. 9).

Caspase-2

It has recently been demonstrated that caspase-2 is essential for the permeabilization of mitochondria and

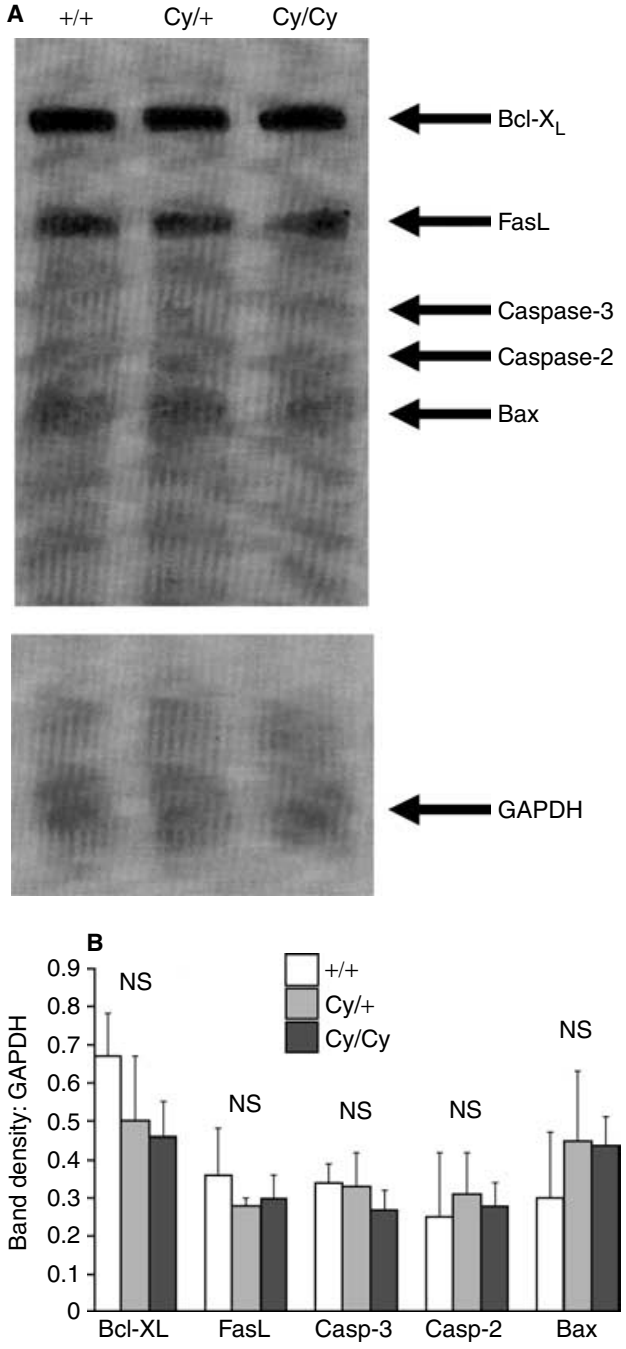

Fig. 3. Ribonuclease protection assay and densitometic analysis. (*A*) Ribonuclease protection assay showed no consistent differences in Bcl-X_L, FasL, caspase-3, caspase-2, and Bax mRNA between 3-week-old homozygous (Cy/Cy), heterozygous (Cy/+), and normal littermate controls (+/+). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control. (*B*) Densitometric analysis of the protected mRNA bands in three separate experiments from three different litters was performed using the computer program Scion Image for Windows that is available on the National Institutes of Health (NIH) website. Results were expressed as the ratio of the band density to GAPDH. No statistical differences in the band density of Bcl-X_L, FasL, caspase-3, caspase-2, and Bax mRNA relative to GAPDH were seen between homozygous (Cy/Cy), heterozygous (Cy/+), and normal littermate controls (+/+). NS is not significant.

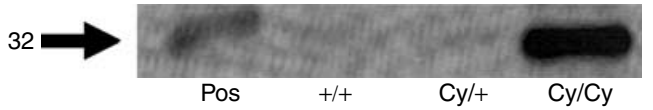

Fig. 4. Immunoblot analysis for caspase-3 in 3-week-old Han:SPRD rat kidneys. On immunoblot for caspase-3, there was an increase of the proform (32 kD) in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys. Positive control (Pos) is purified recombinant caspase-3. Representative immunoblot of three separate experiments from three separate litters.

Fig. 5. Immunoblot analysis for caspase-7 in 3-week-old Han:SPRD rat kidneys. As the fluorescent substrate, Ac-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (DEVD-AMC) (Fig. 2) does not differentiate between caspase-3 and caspase-7 activity, immunoblot analysis was undertaken. There was a massive increase in the proform (35 kD) of caspase-7 in homozygous (Cy/Cy) vs. normal littermate controls (+/+) and heterozygous (Cy/+) kidneys. Caspase-7 was not seen in +/+ and Cy/+ kidneys. Jurkat whole cell lysate was used a positive control (Pos). Representative immunoblot of three separate experiments from three separate litters.

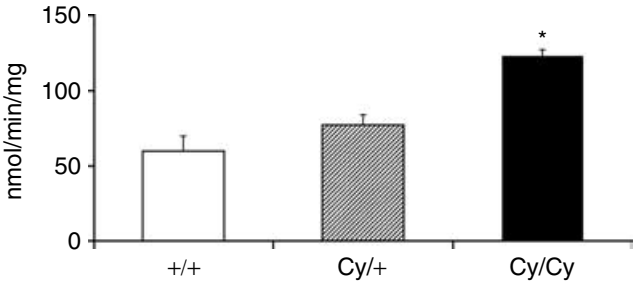

Fig. 6. Caspase-8 and caspase-9 activity in 3-week-old Han:SPRD rat kidneys. Cytosolic extracts of renal cortex were prepared. The activity of caspase-8 and caspase-9 was determined using the fluorescent substrate Ac-Ile-Glu-Pro-Asp-7-Amino-4-methylcoumarin (IEPD-AMC). There was an increase in caspase-8 and caspase-9 activity in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys. $^*P < 0.001$ vs. +/+ and Cy/+ ($N = 10$).

release of cytochrome c [13]. Caspase-2 has two messenger RNAs generated by alternative splicing, which encode caspase-2L and caspase-2S. Although caspase-2L induces apoptosis, caspase-2S has the ability to antagonize cell death [24, 25]. There was no change is caspase-2L mRNA between +/+, Cy/+, and Cy/Cy kidneys (Fig. 3). Caspase-2 activity was increased in Cy/Cy kidneys (Fig. 10). Caspase-2 activity (nmol/min/mg) was 0 in +/+ and 1.75 ± 1.1 in Cy/+ ($P =$ NS vs. +/+) and 5.3 ± 1.2 in Cy/Cy rats ($P < 0.05$ vs. +/+) ($N = 3$ in +/+, $N = 3$ in Cy/+, and $N = 6$ in Cy/Cy) (Fig. 10). Caspase-2 activity was not increased in 8-week-old Cy/+ rats. Caspase-2

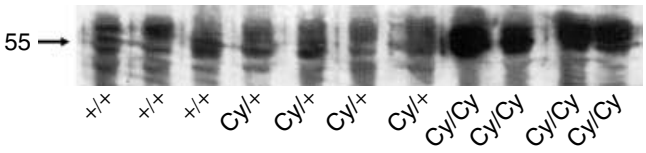

Fig. 7. Immunoblot analysis for caspase-8 in 3-week-old Han:SPRD rat kidneys. As the fluorescent substrate, Ac-Ile-Glu-Pro-Asp-7-Amino-4-methylcoumarin (IEPD-AMC) (Fig. 6) does not differentiate between caspase-8 and caspase-9 activity, immunoblot analysis was undertaken. There was a massive increase in the proform (55 kD) of caspase-8 in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys.

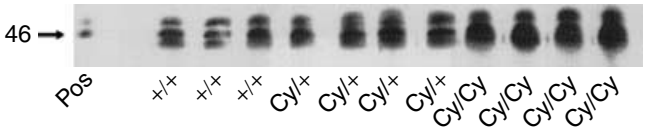

Fig. 8. Immunoblot analysis for caspase-9 in 3-week-old Han:SPRD rat kidneys. As the fluorescent substrate, Ac-Ile-Glu-Pro-Asp-7-Amino-4-methylcoumarin (IEPD-AMC) (Fig. 6) does not differentiate between caspase-8 and caspase-9 activity, immunoblot analysis was undertaken. There was an increase in the proform (46 kD) of caspase-9 in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys. Jurkat whole cell lysate was used a positive control (Pos).

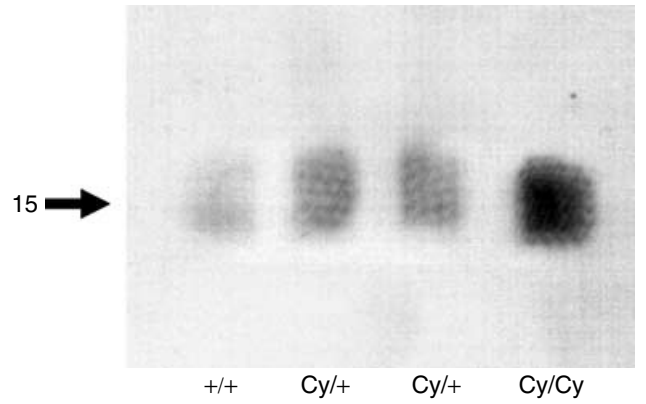

Fig. 9. Immunoblot analysis for cytochrome c in 3-week-old Han:SPRD rat kidneys. Kidneys were rapidly removed from the rats and immediately homogenized. Preparation of cytosolic extracts without disrupting the mitochondria is outlined in the **Methods** section. An increase in cytochrome c (15 kD) in the cytosol was detected on immunoblot analysis in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) kidneys. Representative immunoblot of at least three separate experiments from three separate litters.

activity (nmol/min/mg) in 8 week old rats was 1.9 ± 0.4 in +/+ and 1.3 ± 0.5 in Cy/+ (P = NS vs. +/+) (N = 4). On immunoblot for caspase-2, there was an increase in the proform (45 kD) in Cy/Cy compared to +/+ and Cy/+ rat kidneys (Fig. 11).Caspase-2L mRNA encodes a protein product of 435 amino acids that is homologous to the P20 and P10 subunits of caspase-1 (27% identity) [24]. We also measured caspase-1 activity. Caspase-1 is

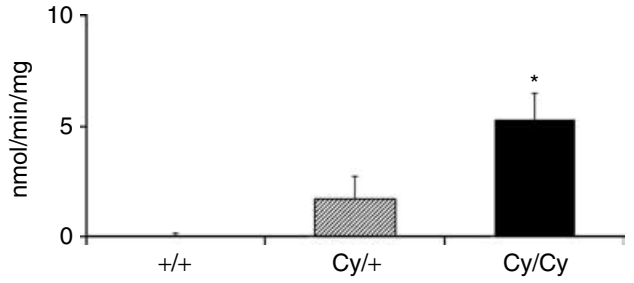

Fig. 10. Caspase-2 activity in 3-week-old Han:SPRD rat kidneys. Cytosolic extracts of renal cortex were prepared. The activity of caspase-2 was determined using the fluorescent substrate Z-Val-Asp-Val-Ala-Asp-Amino-4-methylcoumarin (Z-VDVAD-AMC). There was an increase in caspase-2 activity in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys. *P < 0.05 vs. +/+ and Cy/+, in +/+ (N = 3), in Cy/+ (N = 3), and in Cy/Cy (N = 6).

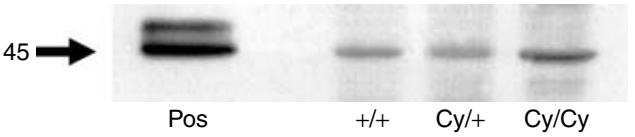

Fig. 11. Immunoblot analysis for caspase-2 in 3-week-old Han:SPRD rat kidneys. On immunoblot for caspase-2, there was an increase of the pro-form (45 kD) in homozygous (Cy/Cy) compared to normal littermate controls (+/+) and heterozygous (Cy/+) rat kidneys. Jurkat whole cell lysate was used a positive control (Pos). Representative immunoblot of five separate experiments from five separate litters.

a proinflammatory caspase that activates the cytokines interleukin (IL)-1 and IL-18. Caspase-1 activity was no different in +/+, Cy/+, and Cy/Cy kidneys. Caspase-1 activity (nmol/min/mg) was 337 ± 69 in +/+ and 377 ± 76 in Cy/+ (P = NS vs. +/+) (N = 5) and 304 ± 51 in Cy/Cy rats (P = NS vs. +/+ and Cy/+) (N = 5).

Fas ligand mRNA

Renal tubular epithelial cells are known to express Fas ligand (FasL) [26]. In the "extrinsic" pathway of apoptosis, the binding of FasL to the death receptor Fas is one of the triggers that leads to the recruitment of caspase-8 and apoptosis [27]. On ribonuclease protection assay there was no difference in FasL mRNA between +/+, Cy/+, and Cy/Cy kidneys (Fig. 3).

Immunohistochemistry for active caspase-3

Kidney sections were stained with an antibody that specifically detects the active form of caspase-3. No staining was detected in +/+ and Cy/+ kidneys (Fig. 12A and B). In Cy/Cy kidneys, staining was detected in tubular cells lining cysts (Fig. 12C) and in the interstitium (Fig. 12D)

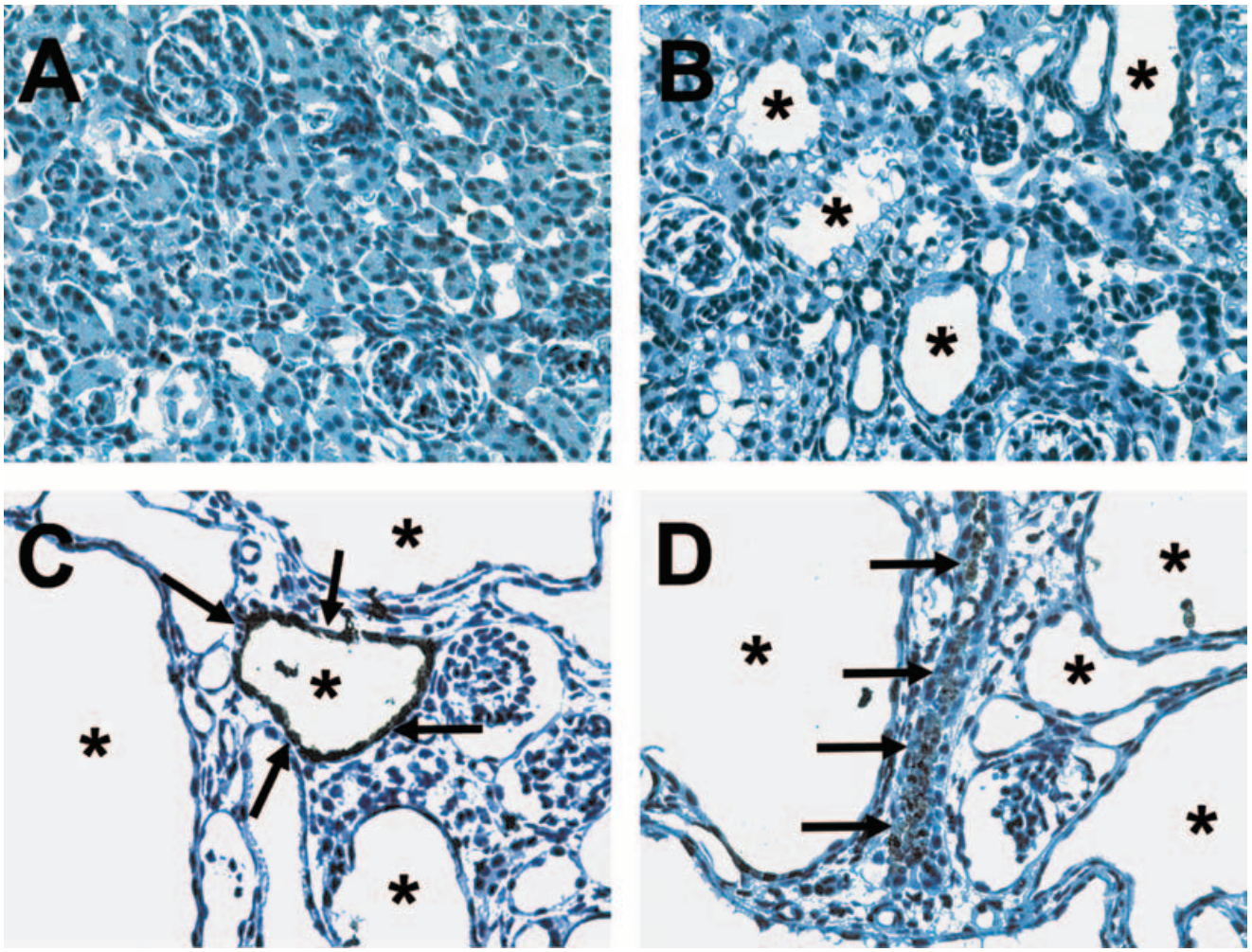

Fig. 12. Immunohistochemistry. Sections were incubated with an antiactive caspase-3 antibody from Promega Corporation (1:50). The antigen site was visualized by incubating the sections with the substrate 3,3′-diaminobenzidine (DAB) chromogen (brown color). No staining was detected in +/+ and Cy/+ kidneys (*A* and *B*). In Cy/Cy kidneys, staining (brown color) (arrows) was detected in tubular cells lining cysts (*C*) and in the interstitium (*D*). No staining was detected in glomeruli in +/+, Cy/+, and Cy/Cy kidneys. Negative control sections showed no staining with DAB. Cysts are marked with an asterisk (*).

Caspase inhibition

The pan caspase inhibitor IDN-8050 (1 mg) or vehicle (PEG 300) was administered intraperitoneally. Caspase-3 and caspase-7 activity was measured in the Cy/Cy kidneys 3 hours after the administration of the inhibitor. Caspase activity in the kidney was 30.5 ± 6.3 in vehicle-treated rats and 4.5 ± 1.5 in IDN-8050–treated rats ($P < 0.05$ vs. vehicle-treated) ($N = 3$) (Fig. 13). Thus intraperitoneal administration of the caspase inhibitor IDN-8050 in the short-term virtually completely inhibits caspase-3 and caspase-7 activity in Cy/Cy kidneys.

The caspase-2 inhibitor Z-VDVAD-FMK was administered intraperitoneally. Caspase-2 activity was measured in the Cy/Cy kidneys 3 hours after the administration of the inhibitor. Caspase-2 activity in the kidney was 5.3 ± 1.2 in untreated rats and 7.5 ± 2.7 in Z-VDVAD-FMK–treated rats (P = NS vs. untreated) ($N = 6$). Thus intraperitoneal administration of the specific caspase-2 inhibitor Z-VDVAD-FMK in the short-term had no effect on caspase-2 activity in Cy/Cy kidneys.

DISCUSSION

In 1989, Kaspareit-Rittinghausen, Deerberg, and Wcislo [28] described a spontaneous mutation in the Sprague-Dawley strain of rats, the Han:SPRD, that inherited PKD as an autosomal-dominant trait [28]. The PKD in this rat has been extensively characterized [14]. Heterozygous animals develop slowly progressive renal cystic disease, whereas homozygotes develop massive cystic enlargement leading to death from uremia at about 4 weeks of age. This rat model exhibits many of the features of ADPKD in humans, including autosomal-dominant inheritance, progression to end-stage renal failure with uremia, hypertension and anemia, and bilateral renal involvement with cysts that derive from all tubular

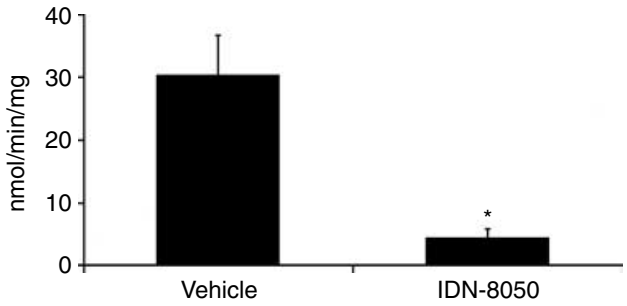

Fig. 13. Short-term caspase inhibition study. The pan caspase inhibitor IDN-8050 (1 mg) or vehicle [polyethylene glycol 300 (PEG 300)] was administered intraperitoneally in homozygous (Cy/Cy) rats. Caspase-3 and caspase-7 activity was measured in the Cy/Cy kidneys 3 hours after the administration of the inhibitor. Caspase activity in the kidney was massively reduced in IDN-8050 compared to vehicle-treated rats. $^*P < 0.05$ vs. vehicle-treated ($N = 6$) and IDN-8050 group ($N = 3$).

segments. We have previously described the presence of increased apoptosis and caspase activity in cystic kidneys of Han:SPRD rats [9]. In the present study, we used this model to further study the molecular pathways of caspase activity and apoptosis in ADPKD.

While apoptosis is a pathologic feature of animal models of PKD [1–4], experimental models of PKD [6–8] and kidneys of humans with ADPKD [3], there have been few studies of the molecular pathways of this apoptosis. In cell culture, the balance between pro- and antiapoptotic Bcl-2 family proteins has been found to control caspase-3 activity by regulating cytochrome c release via the mitochondrial pathway of apoptosis [23, 29]. In support of a role of the mitochondrial pathway of apoptosis in PKD is the demonstration that Bcl-2–deficient mice have increased apoptosis and die from severe polycystic kidney disease [8, 30, 31]. Also, in human kidneys with ADPKD, levels of antiapoptotic Bcl-2 mRNA and protein are markedly elevated [2]. However, in the SBM transgenic mouse model of PKD, both apoptosis and proliferation are c-myc driven and are independent of Bcl-2, p53, and Fasl/Fas interaction [32, 33]. Increased caspase-3 activity and caspase-7 mRNA has been detected in cystic kidneys in congenital polycystic kidney (cpk) mice, a model of ARPKD [18]. In the former study, caspase-4 activity which is known to activate caspase-3 via the extrinsic pathway of apoptosis [34] was also increased. In the present study in ADPKD, we further investigated the individual caspases that are components of the extrinsic and intrinsic apoptosis pathways.

The caspases are a family of intracellular cytoplasmic cysteine proteases. Caspases share a predilection for cleavage of their substrates after an aspartate residue [35]. There are 14 members of the caspase family, caspases 1 to 14. The members of the caspase family can be divided into subfamilies based on substrate specificity and function [19]. Caspase-1 [previously known as IL-1 converting enzyme (ICE)] plays a major role in the activation of proinflammatory cytokines and not in apoptosis. We have demonstated that caspase-1 is a mediator of ischemic acute renal failure in mice [36, 37]. No increase in proinflammatory caspase-1, however, was seen in 3-week-old Han:SPRD rat kidneys.

Caspase-3 plays a crucial and extensively studied role in the promotion of apoptotic cell death [11]. Activation of caspase-3 results in severing of contacts with surrounding cells, reorganization of the cytoskeleton, inhibition of DNA replication, disruption of the nuclear structure, and disintegration of the cell into apoptotic bodies. Increased caspase-3 activity may be due to increased expression of mRNA and/or protein [38]. In the present study, mRNA for procaspase-3 was no different in +/+, Cy/+, and Cy/Cy rats. On immunoblot for caspase-3, there was an increase in the 32 kD proform. The increase in protein without an increase in mRNA suggests that regulation of the protein is post transcriptional and could involve translational control or changes in protein turnover. In this regard, the posttranscriptional regulation of caspase-3 protein was recently described during skeletal muscle development [39].

Caspase-7, like caspase-3, is an "executioner" caspase and is downstream of the "initiators" caspase-8 and caspase-9 (Fig. 1). Both the intrinsic and extrinsic pathways activate caspase-7 (Fig. 1). Caspase-3 and caspase-7 exhibit very similar substrate specificities in peptide hydrolysis assays in vitro [40]. However, the role of caspase-7 during the execution phase of apoptosis is obscure [41]. Caspase-7 is unable to cleave the well-known caspase-3 substrates, including fodrin, gelsolin, DNA fragmentation factor 45 (DFF45), inhibitor of apoptosis proteins (IAP), and signal transducer and activator of transcription-1 (STAT-1) [41]. Also, caspase-7 is unable to activate caspases that would normally be activated by caspase-3 [42]. It is known that caspase-3 and caspase-7 can act independently as "executioners" of apoptosis [43]. Both caspase-3 and caspase-7–deficient mice are perinatally lethal due to a lack of apoptosis [44]. Caspase-7 may play a more specialized role in apoptosis than caspase-3 [41]. In the present study, there was an increase in both caspase-3 and caspase-7 protein suggesting that both these caspases may be involved in ADPKD. The precise determination of which "executioner" caspase is the mediator of ADPKD would require inhibitors that are specific for individual caspases. While the therapeutic efficacy of pan caspase inhibitors has been demonstrated in animal models of disease, in vivo inhibitors of specific caspases have not yet been widely studied.

Next, pathways of caspase-3 and caspase-7–mediated apoptosis were investigated in the kidneys of 3-week-old Han:SPRD rats. The "initiator" caspase-8 and caspase-9 are major players in the "extrinsic" and "intrinsic" pathways of apoptosis, respectively. Caspase-8 and caspase-9 activate the "executioner" caspase-3 and caspase-7. The

critical role of the "initiator" caspase-9 is illustrated in caspase-9 knockout mice that demonstrate the absence of downstream caspase-3 activation [45]. In the present study, there was an increase in both caspase-8 (extrinsic pathway) and caspase-9 (intrinsic pathway) protein. Further investigation demonstrated cytochrome c release, an essential event in the mitochondrial pathway. It is known that there may be an interconnection between the intrinsic and extrinsic pathways via cleavage of the proapoptotic protein, BID, by caspase-8 [46].

Caspase-2 is a recently discovered caspase that is a critical initiator of the mitochondrial apoptosis pathway [13]. Activation and increased activity of caspase-2 is required for the permeabilization of mitochondria and release of cytochrome c [13]. Caspase-2 activity was increased in Cy/Cy rat kidneys. However, mRNA for procaspase-2 was no different in +/+, Cy/+, and Cy/Cy rat kidneys suggesting that the increased caspase-2 activity may be due to regulation of the protein at a translational level or increased stability of the caspase-2 protein.

The death receptors (CD95/Fas/APO-1, TNFR1, DR3/WSL-1/TRAMP, DR4/TRAIL-R1, DR5/TRAIL-R2, and DR6) are a subset of the tumor necrosis factor (TNF) receptor family of cell surface molecules that possess a common motif within their cytoplasmic tails, called the death domain. The death domains of these receptors recruit adapter molecules that, in turn, recruit caspases to the receptor complex. In the extrinsic pathway the death receptors use adapter molecules that recruit caspase-8 [47]. There was no increase in FasL mRNA, an important death receptor ligand in the extrinsic pathway. In normal kidney tubular epithelium, tubules stimulated with inflammatory mediators and in glomerular injury, FasL protein was regulated in concert with mRNA abundance [48]. These data suggest that the extrinsic pathway in ADPKD is independent of the death receptor ligand, FasL.

In the 3-week-old Cy+ rats, the increase in cyst formation precedes the increase in caspase activity. These data suggest that the caspase pathways may not be playing an important role in initial cystogenesis in the Cy/+ rats. In the 8-week-old Cy/+ rats, the increase in caspase activity precedes the increase in serum creatinine. The possible mechanisms by which caspases and apoptosis may contribute to renal failure in polycystic kidney disease are not well understood. An increase in caspase activity and apoptosis localized primarily in the interstitium with little evidence of cell death in cyst epithelium or noncystic tubules has been described in the cpk mouse model of ARPKD [18]. Thus, caspases and apoptosis may destroy the renal interstitium, thereby allowing cystic epithelium to proliferate [49]. In the Cy/Cy rats, the cystic epithelium "survives" as opposed to the non cystic epithelium that virtually vanishes. We have previously described the presence of apoptosis in noncystic tubules [9]. In 6-week-old Cy/+ Han:SPRD rats, 41% of the terminal deoxytransferase (dTd) uridine triphosphate (UTP) nick-end labeling (TUNEL)-positive cells were in noncystic tubules [9]. Thus, it is possible that caspase-mediated apoptosis may lead to the loss of noncystic tubules and renal failure in PKD. However, in the present study, active caspase-3 staining was not seen in 3-week-old +/+ and Cy/+ rat kidneys but was demonstrated in both tubular cells lining cysts and in the interstitium in Cy/Cy rat kidneys. Identification of the cellular pathways involved in apoptosis and the localization of increased caspase activity in the kidney may provide insight into the pathogenesis of cyst formation and renal failure as well as potential therapies in ADPKD.

Pancaspase inhibitors have been used in vivo in animal studies to treat a wide variety of acute disease processes [50]. The therapeutic potential of caspase inhibitors in ADPKD has not been explored. It is not known whether intraperitoneal administration of a caspase inhibitor results in inhibition of caspase activity in the massively enlarged polycystic kidney. Thus, short-term treatment of Cy/Cy rats with a caspase inhibitor was investigated. Intraperitoneal administration of the caspase inhibitor IDN-8050 in the short-term virtually completely inhibits caspase-3 and caspase-7 activity in Cy/Cy kidneys. The effect of long-term administration of these pancaspase inhibitors on apoptosis in cystic and non cystic epithelium and the interstitium as well as cyst formation and mortality in Cy/Cy rats is worthy of study.

CONCLUSION

In 3-week-old Cy/Cy Han:SPRD rat kidneys there is (*1*) increased activity and protein of the "executioner" caspase-3 and caspase-7, (*2*) increased activity and protein of the "initiator" caspase-9 that coincides with an increase in cytochrome c (15 kD) release into the cytosol and an increase in caspase-2 activity demonstrating involvement of the intrinsic pathway, and (*3*) increased activity and protein of the "initiator" caspase-8 suggesting involvement of the extrinsic pathway. In addition, there was no increase in FasL mRNA, suggesting that the extrinsic pathway is independent of the death receptor ligand, FasL.

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